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Although nearly half of human breast cancers at diagnosis are estrogen-responsive and respond to antiestrogen therapy at least for a time, more than half are estrogen receptor negative (ER-) and are predicted to be estrogen-nonresponsive tumors. However, some tumors which are ER- appear to respond to antiestrogen therapy in patients, which is beneficial because antihormonal therapy is less toxic than the alternative chemotherapy. The SCID mouse model has been recently used to study the hormonal dependence of growth of human breast cancer cells as tumors. Recent work has identified a potential model system where ER- breast cancer cells are estrogen-independent in cell culture, and yet intriguingly show estrogen-stimulated growth as tumors in SCID mice. This model system will extend understanding of the host contribution to tumor growth by isolating the host portion of estrogen-stimulated breast tumor growth. The potential to control tumor growth by modulating the host animal mechanism would be of particular value in the treatment of estrogen-nonresponsive tumors which recur after "breakthrough" of tamoxifen therapy, as well as tumors that are ER- and assumed to be estrogen-nonresponsive at diagnosis. Understanding how estrogens can lead to the increased growth of estrogen-nonresponsive breast tumors may lead to *new mechanisms* to better control the growth of estrogen-independent breast cancer.

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Dennis B. Lubahn

PI - Signature

Date

Dec. 19, 1997

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Introduction

Although nearly half of human breast cancers at diagnosis are estrogen-responsive and respond to antiestrogen therapy at least for a time, more than half are estrogen receptor negative (ER-) and are predicted to be estrogen-nonresponsive tumors (1). However, some tumors which are ER- appear to respond to antiestrogen therapy in patients, which is beneficial because antihormonal therapy is less toxic than the alternative chemotherapy. The athymic, *nude* mouse model (2) has been used to study the hormonal dependence of growth of human breast cancer cells as tumors. Recent work has identified a potential model system where ER- breast cancer cells are estrogen-independent in cell culture, and yet intriguingly show estrogen-stimulated growth as tumors in athymic, *nude* mice (3). This model system will extend understanding of the host contribution to tumor growth by isolating the host portion of estrogen-stimulated breast tumor growth. The potential to control tumor growth by modulating the host animal mechanism would be of particular value in the treatment of estrogen-nonresponsive tumors which recur after "breakthrough" of tamoxifen therapy, as well as tumors that are ER- and assumed to be estrogen-nonresponsive at diagnosis. Understanding how estrogens can lead to the increased growth of estrogen-nonresponsive breast tumors may lead to *new strategies* to better control the growth of estrogen-independent breast cancer.

Our working hypothesis is that estrogen regulates breast tumor growth via both direct and indirect stimulatory paths. That is, both tumor cells and host cells, from the body in which the tumor resides, will respond to estrogen and contribute to tumor growth. Friedl and Jordan (3) have reported that estrogen will stimulate growth in estrogen receptor negative breast tumor cells (MDA-MB-231 subclone 10A) that have been implanted in athymic, *nude* mice. The authors proposed that estrogen stimulated normal mouse tissues to produce factors that promoted tumor growth through reduced tumor cell loss rather than through stimulation of tumor cell proliferation even though the cells within the tumor were not directly sensitive to estrogens. Our goal is to confirm this intriguing report by producing an athymic, *nude* mouse that lacks a functional ER and to determine the *in vivo* response of implanted breast cancer cell lines to estrogens.

Finally, we will establish and make available, as a service to the breast cancer research community, ER-minus mice in a *nude* mouse background to be used in model studies to help elucidate the direct and indirect *in vivo* effects of estrogens on breast cancer growth and on progression from estrogen dependence to independence.

Statement of Problem

MDA-MB-231 Subclone 10A cells are an estrogen receptor (ER)-negative, human breast cancer cell line that does not appear to respond to estradiol *in vitro*. Surprisingly, after implantation into athymic, *nude* mice (2), this ER-negative cell line exhibited estrogen-responsive growth as a tumor (3); treatment with estradiol over 9 weeks increased tumor growth (area) up to 10-fold. We propose to study ER- tumor growth in athymic, *nude* mice, in both wild type and ER "knockout" (ER-/-) mice, so as to be able to unambiguously determine the host animal contribution to estrogen-stimulated breast tumor growth. This system represents a unique and valuable model to study the host contribution to hormonal control of breast cancer.

Why is Estradiol Responsiveness in Breast Cancer Tumors Clinically Important?

Breast cancer tumors that are estrogen responsive can be treated with relatively mild antihormonal therapy through the estrogen antagonist tamoxifen. However, most cancers become estrogen-independent, resulting in a resistance to treatment with tamoxifen(1). It would be extremely valuable clinically if we could make a breast tumor regain responsiveness to estrogen and eventually regain its dependence on estrogen. Alternatively, if we knew how a tumor cell lost its capacity to respond to estradiol then we might some day be able to prevent tumor cells from losing responsiveness to hormone treatment.

In this pilot study we will set up a model system that will allow us to study the mechanism by which a breast tumor cell line, lacking both its estrogen receptor and estrogen responsiveness *in vitro,* can exhibit estrogen responsiveness *in vivo*. Suggested mechanisms for this include estrogen responses in the host (not the tumor itself) which lead to increased angiogenesis of the tumor or reduced apoptosis of tumor cells. Our approach will use the ER knockout mouse as a model which will separate host from tumor estrogen responses that lead to increased tumor growth. Understanding how estrogens can lead to the increased growth of estrogen-nonresponsive breast tumors may lead to new strategies to better control the growth of estrogen-independent breast cancer. Finally as a service to the scientific

community, we will establish and make available ER-minus mice in an athymic, *nude* mouse strain background so that both the indirect and direct *in vivo* effects of estrogens on other tumor cell lines can be examined.

Mechanism of ER Action

The estrogen receptor and its ligand, estradiol, have long been thought to be essential for survival (4,5), fertility, and female sexual differentiation and development. A potentially critical time of action for estrogen and its receptor is during blastocyst implantation and early embryogenesis. Support for this possibility was provided in a report by Hou and Gorski (6) in which reverse transcriptase-polymerase chain reaction (RT-PCR) was utilized to demonstrate expression of the ER gene in the mouse during the blastocyst stage of development. Using similar techniques, Wu et al. detected ER mRNA in both human (7) and mouse (8) oocytes, providing evidence for the potential role and importance of ER and estradiol even prior to fertilization. There is also no doubt that estrogen plays a central role in normal postnatal female physiology, as well as in female pathology where its importance in breast and uterine cancer, osteoporosis and cardiovascular disease is well known, although poorly understood.

ER, like all members of the extensively studied steroid receptor superfamily (9-11), is known to act through a now classic mechanism of ligand-dependent transcriptional regulation: 1) synthesis and secretion of estradiol in the ovary; 2) transport of the ligand usually associated with a serum binding protein in the blood stream; 3) passive diffusion through the cell membrane of the target tissues, uterus, breast, bone, etc.; 4) binding of the ligand to the receptor resulting in an allosteric receptor activation; 5) binding of a receptor dimer to a cognate DNA or hormone response element (HRE) usually located in the upstream promoter region of regulated genes; and finally 6) interacting with the transcriptional machinery and modulating transcription of specific genes.

Lack of Classical ER Responses in ER-Minus Mice

Consistent with the proposed crucial roles of estradiol and the ER protein, human ER gene mutations were unknown until recently (12). This is unlike the androgen receptor where many loss-of-function human mutations have been found (13-15). We have been able to generate mutant mice lacking responsiveness to estradiol (ER-minus mice) via gene targeting (16). Exon 2 of the ER gene was disrupted by insertion of a neomycin resistance gene. Both male and female ER- animals survive to adulthood with normal gross external phenotypes. No abnormalities have been detected in heterozygous animals. Homozygous females and males are infertile. Females have hypoplastic uteri and "hyperemic" ovaries with no detectable corpora lutea.

Classic biological assays for estradiol are also unresponsive: In wild type and heterozygous females, three day estradiol treatment with 40 ug/kg dose stimulates a 3 to 4 fold increase in uterine wet weight and alters vaginal cornification, but the uteri and vagina of the ER-disrupted animals do not respond. Prenatal male and female reproductive tract development can therefore occur in the absence of estradiol receptor-mediated responsiveness.

Hypothesis/Purpose:

We propose that cells of host tissue respond to estrogen by producing a "factor" capable of stimulating breast tumor cell growth. Confirmation that host responsiveness to estradiol is required for tumor growth will be sought by implanting the ER-minus breast tumor cell line, MDA-MB-231 (subclone 10A), in *nude* ER-minus mice lacking estrogen responsiveness. If a host response to estradiol is required for tumor growth then the tumor will not respond in ER-minus mice.

The lack of ER-negative tumor response to estradiol in the ER-minus mice provides support for these two possible mechanisms:

1. The breast tumor cells are responding indirectly to estrogen, perhaps through a factor induced in another tissue in the mouse that is altering tumor cell proliferation and/or apoptosis.
2. Estradiol increases the rate of angiogenesis and nutrient supply thus allowing faster tumor growth.

If, as we expect, the ER-negative tumor cells are non-responsive to estradiol *in vivo*, then the case for direct stimulation by the following mechanisms is weakened:

1. The tumor cells regain functional estrogen receptor protein *in vivo*.

2. One of the other known non-ER, estrogen binding proteins, perhaps capable of mediating a growth response, is induced *in vivo*.

Technical Objectives:

Technical Objective #1.

We will establish a colony of athymic, *nude* mice and ER+/- mouse heterozygotes to produce 5-15 animals per week of female athymic, *nude* ER-/- mice and control female athymic, ER+/+ mice sufficient for the technical objectives.

Technical Objective #2.

A. While we are establishing the colony of athymic, *nude*, ER-minus mice, we will use commercially available athymic, *nude* mice (ER-positive) to confirm that the estrogen receptor-negative MDA-MB-231, subclone 10A, breast cancer cell line will not respond to estrogen in *in vitro* culture but will respond *in vivo* in the athymic, *nude* mouse. We will use estrogen-dependent MCF-7 cells as positive controls for estrogen responsiveness and parental MDA-MB-231 cells as controls for estrogen-independence. (See Table 1) Both of these human breast cancer cell lines have been used extensively in the athymic mouse model. We expect that MCF-7 cells will grow as tumors that require estrogen in the athymic mice, and that parental MDA-MB-231 cells will grow as tumors independent of estrogens. Furthermore, we expect that estrogens will increase the growth of the subclone 10A cells only in the ER-positive mice, confirming the results of Friedl and Jordan (3). We will also test the specificity of this response with estrone, estriol, tamoxifen, the antiestrogen, ICI 164,384 and catechol estrogen to determine if the response is mediated by ER protein of the host animal.

B. We will confirm that the subclone 10A tumors lack estradiol binding activity at the end of the experiments, as well as ER protein and mRNA both *in vitro* and *in vivo*. We will assay for ER protein by estradiol binding activity and Western blot analysis and assay for mRNA by RNase protection and quantitative polymerase chain reaction (PCR) analysis. We expect that the tumors will lack ER in any form both *in vitro* and *in vivo*.

Another possible result, almost as intriguing is that the tumors possess ER protein or mRNA either *in vitro* or *in vivo*, but it is only functional *in vivo* or it is only induced *in vivo*. (We consider this unlikely but if it happens See Future Directions below.)

Technical Objective #3.

We will test the hypothesis that the ER-negative tumor cell line, MDA-MB-231 subclone 10A, is being indirectly influenced by factors induced by estradiol acting through ER protein in tissues outside the tumor. We will implant the ER-minus tumor cell line into a female *nude*, ER-minus mouse. (See Table 1) We have already made an ER-minus mouse lacking a functional ER protein by disrupting the ER gene by homologous recombination. We will cross this ER-minus mouse with an athymic, *nude* mouse on a C57BL background. The resulting athymic, ER-minus mouse will allow the implantation of exogenous tumor cells into a mouse lacking responsiveness to estradiol. We can then determine whether the growth response of the tumor cells to estradiol is mediated directly or indirectly by estradiol. If our hypothesis is correct, the *in vivo* tumor cell line should not respond to estradiol in an environment lacking a functional ER. Further characterization of the putative growth factors could then be undertaken by methods briefly outlined in Future Directions below. A positive response to estradiol would suggest that a search should be begun, both within the tumor cell line and within the ER-minus mouse, for a novel non-ER, estrogen response protein.

We will use estrogen-dependent MCF-7 cells as positive controls for estrogen responsiveness and parental MDA-MB-231 cells as controls for estrogen-independence. Both of these human breast cancer cell lines have been used extensively in the athymic, *nude* mouse model. We expect that MCF-7 cells will grow as tumors that require estrogen in both ER-positive and ER-minus athymic mice, and that parental MDA-MB-231 cells will grow as tumors independent of estrogens in both mice. However, we expect that estrogens will increase the growth of the subclone 10A cells only in the ER-positive mice.

Technical Objective #4.

From the estrogen-responsive MCF-7 human breast cancer cell line, we have derived several unique clonal sublines which are estrogen-independent for proliferation in cell culture (17). We will screen these cell lines for estrogen-dependence of tumor growth in the athymic, *nude* ER-minus mouse model which we have described above. (See Table 1)

The goal is to identify other cells lines which may contribute to the study of the estrogen-stimulated host response and test the generality of ER-minus tumors responding to estrogens.

ER-Minus Mice PCR analysis, RNase protection, Western Northern and Southern Nucleic Acid Analysis to Check for Functional Estrogen Receptor

Western blot protein analysis does not detect any classic ER protein in ER-minus mice. Northern blot analysis of poly A-mRNA isolated from ER minus uteri and probed with either a radioactive ER cDNA probe or neomycin gene probe detected the same size band. Southern blot analysis with ER and neomycin probes shows the expected 2 kb increase in size of the band from the ER gene because of the insertion of the 2 kb neomycin resistance gene. The presence of a single band with neomycin and ER probes indicates that there is only one insertional disruption integration site and that it is in the ER gene.

RNase protection studies with ligand-binding domain probes reveal that the ER-minus mice contain less than 20% of the level of mRNA in this region when compared to wild type ER mRNA. No mRNA containing the full length wild type sequence is detected by PCR although small incomplete mRNA fragments that use cryptic and native donor/acceptor splice sites are detected indicative of neomycin gene disruption of the ER gene transcript. This indicates as expected that some disrupted ER mRNA is being made. It is inactivated as expected by the inserted neomycin gene, because no classic ER protein functional responses are detected.

ER-Minus Mice Biological Responses

We have been concerned that ER protein might somehow be present by "leakage" in the ER-minus mice. Several experiments on the ER-minus mice phenotype in classic estrogen response assays have been performed since the publication of the original ER-minus mouse paper (16,18). We have been unable to detect any classic estradiol responses utilizing several classic biological estrogen assays and transcription-mediated assays in these ER-minus animals.

The biological assay results showing a lack of estradiol response are listed below:

1. Estradiol, as found previously, has reproducibly been unable to increase uterine wet weight and vaginal cornification.

2. Previously it has been found that uteri would respond to epidermal growth factor (EGF) mediated by ER protein but independent of estradiol. There was no response to EGF in these mice, again indicating the absence of functional ER protein.

3. Tamoxifen and diethylstilbestrol (DES) have estrogenic activity in the uterine wet weight gain assay in wild type animals. No effect on uterine wet weight was seen after administration to the ER-minus animals. It has been speculated that there are alternative steroid receptor super family orphan receptors, in addition to ER, for tamoxifen and DES. If these additional receptors exist, they are not able to function to increase uterine wet weight.

4. In other estradiol biological response assays in ER-minus mice, estradiol is also unable to show any response. No increased response was seen after estradiol administration in: a) in ^{3}H-thymidine incorporation in in vitro uterine uptake assays, b) in PR mRNA levels, c) in glucose 6-phosphate dehydrogenase mRNA levels, and d) in lactoferrin mRNA levels. These are very sensitive transcriptionally-mediated, classic estradiol response assays. The lactoferrin mRNA assay, for example, shows over a 350 fold stimulation in wild type animals after exposure to estradiol.

5. Very recent results within the last week have shown by in situ hybridization assays that 12 hour exposure to 4-hydroxy catechol estrogen but not estradiol can induce lactoferrin mRNA in ovariectomized ER-minus mice uteri. This result provides support for the existence of at least one additional "estrogen" response protein capable of responding to an estrogen "metabolite".

ER-Minus, Estrogen-Nonresponsive Cell Lines Derived from the Estrogen-Dependent MCF-7 Human Breast Cancer Cells

The MCF-7 human breast cancer cell line is widely used as a model of hormone-responsive breast cancer. These cells contain ER, progesterone receptors (PgR), and show estrogen-dependent cell proliferation. By culture under hormone-free conditions, Wade Welshons' laboratory has isolated two intermediate cell lines in progression from the ER+, PgR+, estrogen-responsive phenotype of the parental MCF-7 cells, to the ER-, PgR- and estrogen-nonresponsive *in vitro* phenotype (17). This progressive series of cell lines follows the sequence: loss of PgR, loss of estrogen-responsive proliferation with reduced ER, and finally complete loss of ER. Several subcloned cell lines of each phenotype have been

isolated. All ER-minus cell lines fail to express ER mRNA by the RNase protection assay, but all cell lines have an apparently intact ER gene by genomic Southern analysis (17). In Technical Objective #4, we propose to determine if any estrogen-nonresponsive clones in this series will, like subclone 10A proposed for extensive study above, exhibit estrogen-stimulated growth as tumors in athymic mice. This will test the generality of the phenomena that estradiol can exert positive effects on tumor growth through host mediated action.

Table 1

Expected Growth Responses to Estrogen Treatment of Breast Cancer Cells (with and without ER) Implanted in ER wt & ER - athymic, *nude* Mice

	A	*B*	*C*	*D*
	MCF-7 (ER-positive)	MDA (ER-minus)	Subclone 10A (ER-minus)	Our MCF-7 clones (ER-minus)
Nude ER wt/wt mice				
no E2	–	+++	+	+ (?)
with E2	+++	+++	+++	???
Nude ER -/ - mice				
no E2	–(?)	+++(?)	+(?)	+ (?)
with E2	++(?)	+++(?)	+(?)	+ (?)

Brief description of breast cancer cells above that are to be implanted into ovariectomized mice for tumor growth experiments:

A. MCF-7: ER-positive, estrogen-responsive for proliferation in culture and as tumors

B. MDA-MB-231 parental: ER-minus, estrogen-nonresponsive for proliferation in culture and as tumors

C. Subclone 10A: Derived from MDA-MB-231; ER-minus, estrogen-nonresponsive for proliferation in culture, but estrogen-responsive for growth as tumors

D. MCF-7 clones (several): ER-minus, estrogen-nonresponsive for proliferation in culture, estrogen-responsiveness unknown as tumors (Isolated in Wade Welshons' laboratory by Ed Curran.)

ER Reactivation in *in vitro* ER-negative tumors

The detection of ER protein and mRNA in the breast tumor cell lines we are examining would raise two more questions (at least!).

A. If the all of the human tumor cell lines really contain ER both *in vitro* and *in vivo*, then the question becomes: "Why does the cell respond *in vivo* and not *in vitro*?" or restated:
"Are additional growth factors from the mouse necessary for estradiol to induce a response?" (See above.)

B. If the tumor cell line only contains ER *in vivo* but not *in vitro*, then the question becomes: "What is the mechanism by which ER protein is induced *in vivo* so that cells regain their capacity to respond to estradiol *in vivo* but not *in vitro*?" Recently it has been found that the inactive ER gene expression can be induced *in vitro* by changes in DNA methylation (27,28). It will be extremely interesting to see if a growth or host factor is changing ER gene expression perhaps by an *in vivo* change in ER gene methylation.

Body
Methods:

D. Statement of Work

Technical Objective #1
Establishment of a Breeding Colony of Athymic, *Nude* ER-Minus Mice
Plan:
Colony will be up to size by end of first year of funding and be maintained through end of grant period.

The crossing of the ER-minus mouse into the athymic, *nude* background will require four generations before we can routinely produce ER-minus, *nude* female mice capable of accepting breast tumor xenografts Because the *nude* mice are immune compromised, all breeding with these mice will be done under microisolater conditions with everything coming in contact with the animals being first autoclaved. The animals will be taken out of their microisolaters only under a laminar flow hood to help maintain sterility. Genotyping of the mice pups will be by appearance for the *nude* genotype and by multiplex PCR for the ER-minus genotype. Multiplex PCR genotyping is routinely done for our ER-minus colony on about 200 DNA samples per week, isolated from tail snips of five week old mice. This same procedure and scale will be followed for the *nude*, ER-minus mouse colony.

In the first generation two *nude* C57BL/6J males, obtained from Jackson Laboratories, will be crossed with new pairs of heterozygous ER-minus female mice every two weeks. (See Figure 1) (The C57BL/6J strain background was chosen because it is the strain into which our ER-minus colony is being backcrossed to remove the 129 background of the original male founder.) Female *nude* mice are poor mothers so they are not useful in efficiently generating offspring. These multiple initial matings will efficiently generate double heterozygous ER-minus/*nude* females at a 1 in 8 frequency. (This frequency is the best possible whenever dealing with ER-minus crosses because heterozygotes must be used yielding only 1 in 4 ER-minus animals per mating, of which only half are the desired sex.) In the second generation the double heterozygote females will be crossed with the males of the same genotype as their father. This backcross will generate both female offspring with the same genotype as the mother (frequency of 1/8) and male *nude*, heterozygote ER-minus offspring (frequency of 1/8). These two genotypes will be crossed in the third generation to produce female *nude*, ER-minus mice at a frequency of 1 in 16. Their siblings will be used to set up additional breeding pairs to produce additional female *nude*, ER-minus mice. It is estimated that 40 litters of 8 pups each will be necessary to produce the 20 female ER-minus, *nude* mice necessary for a typical experiment.

Results:

Progress so far has been frustrating. The success of this objective is dependent on the successful breeding of sufficient numbers of female *nude*/ERαKO mice to be immune compromised recipients of xenotransplants of human breast cell lines to determine their responses to estrogens. This objective is essential for the project to succeed. Unfortunately because of two main findings we have had to alter our approach to this objective. Our first finding was that litter size has been half that expected. Our second finding was that the ratio of male to female pups was 2:1. While these may be from a basic science point of view fascinating, they have hindered the accomplishment of this objective. These two findings have taken our expected ratio of 1 in 16 pups (to be the correct sex and genotype) down to about 1 in 32 in twice the number of litters. As a result we have only been able to breed less than 10 mice of the correct genotype and sex and these have been of unmatched ages which have not allowed us to run controlled experiments.

Fortunately, we have developed and begun to implement a new approach, which should overcome our problems with using *nude* mice. This is to substitute SCID (severe combined immuno-deficient) mice in a C57BL/6J background obtained from Jackson Laboratories. These animals have recently begun to be used in other laboratories to replace *nudes* in breast cancer xenotranplantation studies. They will be important because unlike the *nude* mice, they are more prolific breeders and more importantly the homozygous females are fertile. This should allow us to approach a 1 in 8 frequency.

Technical Objective #2
Results:

The discovery of a second estrogen receptor, ER-beta (17b), as well as our own work that a third estrogen receptor (ER-gamma?) may exist (17c), has confounded this objective. We have developed PCR primers to check for both

ER-α, and ER-beta mRNAs to clarify the true of ER status of published "ER-negative" human breast cell lines. These studies are ongoing.

Technical Objective #3
Results:

No ERaKO/*nude* mice so no results yet. See Objective #1 results above.

Technical Objective #4
Results:

With research progressing so slowly on the first three objectives we have turned our attention to this objective. We have analyzed the MDA cell line and the MCF-7 cell lines developed by Curran and Welshons (See Figure 1) and have 2 significant results to report from analyzing these lines

One of the accepted dogmas for the inactivation of ER in breast tumors, so that it is no longer responsive to estrogens, has been that when DNA methylation occurs at the NotI site of the ER gene, its transcription is turned off. Thus methylation and perhaps demethylation might allow ER-negative tumors to be reactivate their ER genes and subsequent responsiveness. We have shown that in these MCF cell lines there is not a correlation of methylation and ER expression (Cancer Research *submitted*). In the process of looking for correlation of methylation at other DNA sites of expression of the ER gene we have developed a technique that allows for the entire genome to be scanned to look for differences in DNA methylation (Ref 17d Huang *et al.*; See Appendix for this Cancer Research paper). In particular this exciting technique has allowed the discovery of a novel tumor suppressor gene (WT1) which is apparently inactivated in breast cancer. The role of estrogen in regulating this tumor suppressor is at present unclear.

If ER negative cell lines are becoming responsive to estrogens then perhaps it is not ER-alpha that is being reactivated but rather ER-beta or another ER. Our second significant result is the utilization of an RT-PCR assay to analyze for ER-beta mRNA in these MDA and MCF-7 cell lines (Figure 1). The data presented in Figure 2 (lanes 1, 4, 7, 10, 13, 16, 19, 22) clearly shows that ER-beta mRNA is not present in any of the breast cancer cell lines but is present in the positive control human testes RNA (Figure 2 lane 25). Small bands in these lanes are non-specific primer dimers. Other controls show that RNA is present in all samples because actin and RLP7 mRNAs are present in all samples.

Figure 1.

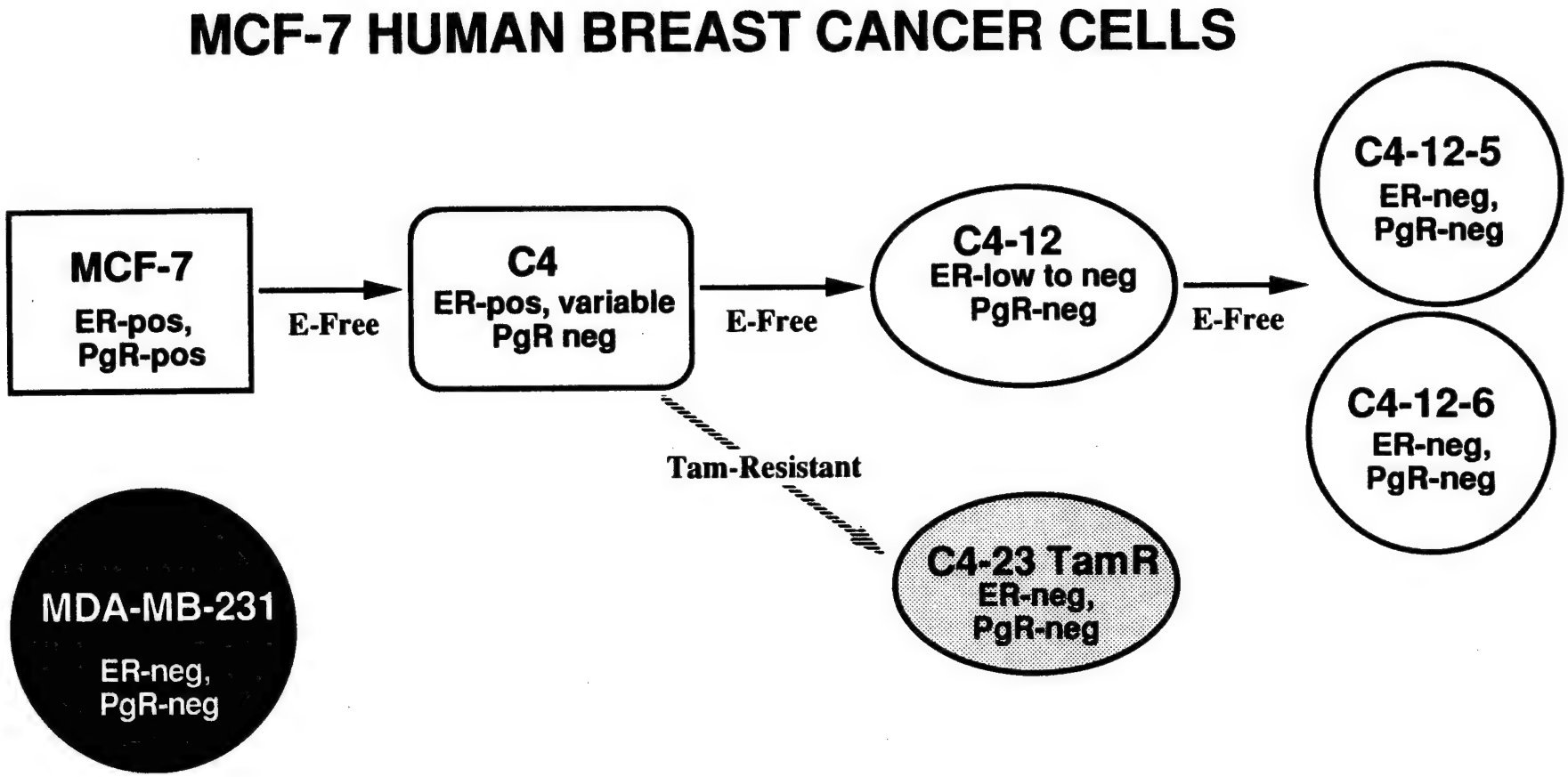
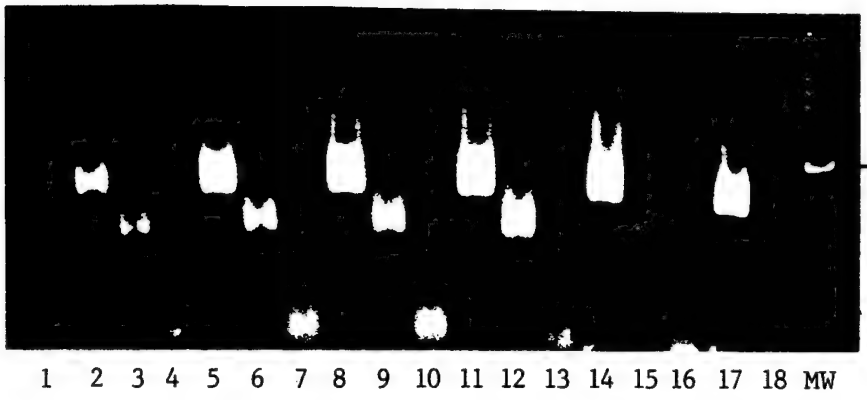
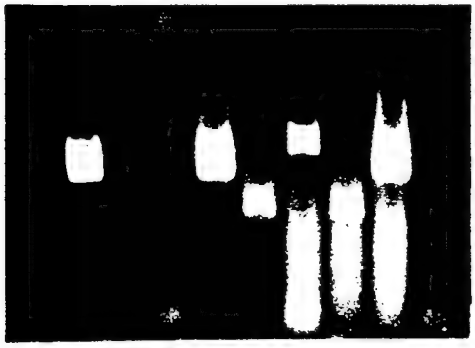

MCF-7 HUMAN BREAST CANCER CELLS
MCF-7
ER-pos,
PgR-pos
E-Free
C4
ER-pos, variable
PgR neg
E-Free
C4-12
ER-low to neg
PgR-neg
E-Free
C4-12-5
ER-neg,
PgR-neg
C4-12-6
ER-neg,
PgR-neg
Tam-Resistant
C4-23 TamR
ER-neg,
PgR-neg
MDA-MB-231
ER-neg,
PgR-neg

Figure 2 Legend. RT-PCR Analysis for ER-beta mRNA in Breast Cancer Cell Lines with Varying amounts of estradiol binding activity.

PCR amplification was performed on each total RNA from the cell lines shown in Figure 1 utilizing three primer sets in the following order: a. Human ER-beta primers 1085 and 1499; b. Actin primers; c. rpL7 primers

Lanes 1-3	Total RNA from MCF 7 Negative for ER alpha
Lanes 4-6	Total RNA from MCF7 Positive for ER alpha
Lanes 7-9	Total RNA from MDA-MB-231
Lanes 10-12	Total RNA from C4
Lanes 13-15	Total RNA from C4-12
Lanes 16-18	Total RNA from C4-12-5
Lanes 19- 21	Total RNA from C4-12-6
Lanes 22-24	Total RNA from C4-23 TamR
Lanes 25-27	Total RNA from Human testis RNA
MW lanes	100 base pair DNA Molecular Weight Markers





Conclusions:

We have successfully developed assays and analyzed for the recently discovered ER-beta mRNA (17b). These assays will allow us to analyze tumors for *in vivo* activation of this receptor as well as ER-alpha. We have recently discovered evidence for a potential third "ER-gamma" (17c) that responds to catechol estrogens but not estradiol. We will thus need to analyze for differential responses of estradiol *vs.* catechol estrogens.

DNA methylation assays have been developed to analyze the ER gene, as well as other DNA methylations throughout the genome (Ref 17d; See paper in Appendix). This will allow us to look for non-mutational activations of the ER gene IF this methylation site is found in the remaining time of this grant.

Finally, a potentially better and more valuable new mouse model, the SCID/ERαKO mouse, is being developed for the scientific community to allow the study of estrogens and xenoestrogens in breast cancer. This will replace the technically unusable *nude*/ERαKO mouse model proposed in our original study.

References

1. Jordan VC, Murphy CS Endocrine pharmacology of antiestrogens as antitumor agents. Endocrine Reviews 11:578-610 (1990).
2. Shafie SM, Grantham FH Role of hormones in the growth and regression of human breast cancer cells (MCF 7) transplanted into athymic *nude* mice. Journal National Cancer Institute 67:51-56 (1981).
3. Friedl A, Jordan VC Oestradiol stimulates growth of oestrogen receptor-negative MDA-MB-231 breast cancer cells in immunodeficient mice by reducing cell loss. European Journal of Cancer 30A 1559-1564 (1994).
4. George, F.W., Wilson, J.D. 1988 In: (Knobil E, Neil JD, Ewing LL, Greenwald GS, Markert, C.L., and Pfaff DW, eds) *The Physiology of Reproduction*, Raven Press, Ltd., New York, pp 3-26
5. Korach KS 1994 Insights from the study of animals lacking functional estrogen receptor. Science 266:1524-1527
6. Hou, Q., and Gorski, J. (1993). Estrogen receptor and progesterone receptor genes are expressed differentially in mouse embryos during preimplantation development. Proc. Natl. Acad. Sci. USA, 90:9460-9464.
7. Wu T-C J, Wang L, and Wan Y-J Y (1993) Detection of estrogen receptor messenger ribonucleic acid in human oocytes and cumulus-oocyte complexes using reverse transcriptase-polymerase chain reaction. Fertility and Sterility 59:54-59.
8. Wu T-C J, Wang L, and Wan Y-J Y (1992) Expression of estrogen receptor gene in mouse oocyte and during embryogenesis. Mol Reprod Dev 33:407-412.
9. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889-895
10. Moore DD 1990 Diversity and unity in the nuclear hormone receptors: A terpenoid receptor superfamily. New Biologist 2:100-105
11. Auchus RJ, Fuqua SAW 1994 The oestrogen receptor. Bailliere's Clin Endocrin Met 8:433-44
12. Smith, E.P., Boyd, J., Frank, G.R., Takahashi, H., Cohen, R.M., Specker, B., Williams, T.C., Lubahn, D.B., and Korach, K.S. 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. New England Journal of Medicine 331:1056-1061
13. Lubahn DB, Brown TR, Simental JA, Higgs HN, Migeon CJ, Wilson EM, French FS 1989 Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. Proc. Nat. Acad. Sci. USA 86:9534-9538
14. Brown TR, Scherer PA, Chang YT, Migeon CJ Ghirri P, Murono K, Zhou Z 1993 Molecular genetics of human androgen insensitivity. Eur J Ped 152 Suppl 2:S62-9
15. Wilson JD, Griffin JE, Leshin M, MacDonald PC 1983 The androgen resistance syndromes: 5a-Reductase deficiency, testicular feminization, and related disorders. In: (Stanbury, J.B., Wyngaarden, J.B., Fredrickson DS, Goldstein JL, Brown MS, eds) *The Metabolic Basis of Inherited Disease*, McGraw-Hill, New York pp 1001-1026

16. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, and Smithies O. 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proceedings of the National Academy of Sciences USA 90:11162-11166.
17. Curran, E.M., Judy, B.M., Golomb, M. and Welshons, W.V., 1994. A hormone-independent, estrogen-receptor-negative cell line isolated from MCF-7 human breast cancer cells: relation to tumor progression. Endocrinology 134 (Suppl): 390, Abstract 757.
17b. Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson, J-A Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925-5930 (1996).
17c . Responses to estrogens in estrogen receptor-alpha minus mice. Das SK. Dey SK. Taylor JA. Lubahn DB. Proceedings of the National Academy of Sciences USA *in press* 1997.
17d Huang TH, Laux DE, Hamlin B, Tran P, Tran H Lubahn DB. Identification of DNA methylation markers for human breast carcinomas using the methylation-sensitive restriction fingerprinting technique. Cancer Res 57:1030-4 (1997).
18. Couse, J.F., Curtis, S.W., Washburn, T.F., Golding, T.S., Lubahn, D.B., Smithies O. and Korach, K.S. The estrogen receptor "knockout" mouse: Analysis of transcription and estrogen insensitivity in the female. Molecular Endocrinology 9 #11(1995).
19. Welshons, W.V., Grady, L.H., Engler, K.S. and Judy, B.M., 1992. Control of proliferation of MCF-7 breast cancer cells in a commercial preparation of charcoal-stripped adult bovine serum. Breast Cancer Res. Treat. 23: 97-104.
20. Ferre F, Marchese A, Pezzoli P, Griffin S, Buxton E, and Boyer V 1994 In:(Mullis KB, Ferre F, Gibbs RA, eds) The Polymerase Chain Reaction Birkhauser, Boston, pp 67-88
21. Gottardis MM, Jiang SY, Jeng MH, Jordan VC 1989 Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Research 49:4090-4093.
22. Morales DE, McGowan KA, Grant DS, Maheshwari S, Bhartiya D, Cid MC, Kleinman HK, Schnaper HW Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model Circulation 91:755-763(1995).
23. Cunha GR Role of mesenchymal-epithelial interactions in normal and abnormal development of the mammary gland and prostate. Cancer 74:1030-44(1994).
24. Cardiff RD, Munn RJ Comparative pathology of mammary tumorigenesis in transgenic mice. Cancer Letters 90:13-19 (1995).
25. Soto AN, Sonnenschein C Cell proliferation of estrogen-sensitive cells: The case for negative control. Endocrine Reviews 8:44-52 (1987).
26. Liang, P. and Pardee, A.B. 1992 Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257: 967-971
27. Ferguson AT, Lapidus RG, Baylin SB, Davidson NE Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. Cancer Research 55:2279-2283 (1995).
28. Weigel RJ, deConinck EC Transcriptional control of estrogen receptor in estrogen receptor-negative breast carcinoma. Cancer Research 53:3472-3474 (1993).

Appendices

See attached Cancer Research paper: Huang *et al.* 1997. (Reference 17)

[CANCER RESEARCH 57, 1030–1034, March 15, 1997]

Advances in Brief

Identification of DNA Methylation Markers for Human Breast Carcinomas Using the Methylation-sensitive Restriction Fingerprinting Technique[1]

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Abstract

We have developed a PCR-based method, called methylation-sensitive restriction fingerprinting (MSRF), to screen changes in DNA methylation in breast carcinomas. Two hypermethylation-containing fragments, HBC-1 (for "hypermethylation in breast cancer") and HBC-2, were identified in the amplified breast tumor DNA relative to the amplified normal breast DNA of a patient. Nucleotide sequence analysis revealed no significant matches between the sequence of HBC-1 and the known sequences in the GenBank database, whereas the sequence of HBC-2 matched the upstream region of an antisense *WT1* (Wilms' tumor suppressor gene) promoter. The methylation status in the breast tumor DNA from this patient was confirmed by Southern hybridization using HBC-1 and HBC-2 as probes, respectively. Further analysis showed that HBC-1 was methylated aberrantly in 90% (17 of 19 patients) of the primary breast carcinomas examined. This study demonstrates that MSRF provides a useful means for screening aberrant changes in DNA methylation during tumorigenesis. The commonly methylated fragments identified by MSRF could potentially supplement pathological markers currently used for cancers and additionally lead to the discovery of novel methylated tumor suppressor genes.

Introduction

Abnormal changes in DNA methylation have been observed in tumor cells (for a review, see Refs. 1 and 2). These changes usually occur in CpG dinucleotides that are clustered frequently in regions of about 1–2 kb in length, called CpG islands, in or near the promoter and first exon regions of genes (3). Hypermethylation within CpG islands was found to be associated with inactivation of tumor suppressor genes, including the *estrogen receptor* gene (4) and the *MDGI* (mammary-derived growth inhibitor) gene (5) in breast carcinoma, the *glutathione S-transferase* gene in prostate carcinoma (6), the *von Hippel-Lindau* tumor suppressor gene in renal cell carcinoma (7), the *RB* gene in retinoblastoma (8), and the *hypermethylation in cancer 1* gene (9), the *cyclin-dependent kinase N2/p16* gene (10, 11), and the *E-cadherin* gene (12) in several types of tumor cells. In addition, aberrant hypermethylation may contribute to tumorigenesis through the C-to-T mutation at the methylated CpG dinucleotides (2). Hypomethylation was also observed in nucleotide sequences of several proto-oncogenes. Potentially, hypomethylation is linked to aberrant increases in the transcription of these genes. However, a direct correlation between hypomethylation and the up-regulation of proto-oncogene expression in tumor cells has yet to be established. As a further step to understanding the role of DNA methylation in tumorigenesis, abnormally hypermethylated or hypomethylated sequences occurring in the tumor genome need to be identified.

In this study, we have developed a PCR-based method, called MSRF,[3] to identify abnormally methylated CpG sites in breast carcinomas. Initially, genomic DNA was digested with a 4-base restriction endonuclease known to cut bulk DNA into small fragments but rarely to cut in the CG-rich regions. The digests were treated with a second restriction endonuclease, which discriminates between methylated and unmethylated CpG sites, and then amplified by PCR with short arbitrary primers (10-mers) at low stringency. Amplified products or DNA fingerprints were resolved by high-resolution gel electrophoresis, and aberrantly methylated patterns were detected in the amplified tumor DNA relative to the amplified normal DNA of the same patient. Here we have successfully employed the technique to identify and clone genomic fragments that frequently undergo methylation changes in primary breast carcinomas.

Materials and Methods

Tissue Samples. Tumor specimens were obtained from patients with invasive breast carcinomas undergoing mastectomies or biopsies at the Ellis Fischel Cancer Center (Columbia, MO; this study has been approved by our institutional review board). The adjacent nonneoplastic breast tissue was also obtained from the same patient to serve as a control (designated as "normal"). High molecular weight DNA was extracted using standard methods.

MSRF. Genomic DNA was digested with *Mse*I alone or digested with *Bst*U I and *Mse*I at 10 units per μg DNA following the conditions recommended by the supplier (New England Biolabs). The PCR reaction was performed with the digested DNA (20–100 ng) in a 20-μl volume containing a pair of primers (0.4 μM), 0.15 units of Taq DNA polymerase (Life Technologies, Inc.), 5% (v/v) DMSO, 200 μM deoxynucleotide triphosphates, and 2 μCi [α-^{32}P] dCTP (3000 Ci/mmol; Amersham) in buffer III [30 mM Tricine (pH 8.4), 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.01% (w/v) gelatin, and 0.1% (w/v) thesit] (13). The primers were as follows: Bs1, 5′-AGCGGCCGCG; Bs5, 5′-CTCCCACGCG; Bs7, 5′-GAGGTGCGCG; Bs10, 5′-AGGGGACGCG; Bs11, 5′-GAGAGGCGCG; Bs12, 5′-GCCCCCGCGA; Bs13, 5′-CGGGGCGCGA; Bs17, 5′-GGGGACGCGA; and Bs18, 5′-ACCCCACCCG. Initial denaturation was for 5 min at 94°C. DNA samples were then subjected to 30 cycles of amplification consisting of 2 min of denaturation at 94°C, 1 min of annealing at 40°C, and 2 min of extension at 72°C in a PTC-100 thermocycler (M. J. Research, Inc.). The final extension was lengthened to 10 min. The products (20 μl) of each amplification reaction were mixed with 5 μl of loading dye. Four μl of each DNA sample were size fractionated on a 4.5% nondenaturing polyacrylamide gel. Samples were loaded onto the gel at ~16 V/cm to prevent diffusion from each lane into its neighboring lanes. The gel was then run for 2 h at 60 W until the bromphenol blue dye front reached 5–10 cm from the bottom edge of the gel. After electrophoresis, wet gels were wrapped with plastic film and subjected to autoradiography at −70°C for 24–48 h with DuPont Ultra-vision film.

Cloning and Sequencing. Target bands were excised from polyacrylamide gels, and gel fragments were shattered by being centrifuged through a pinhole of a microfuge tube into a second intact microfuge tube. DNA was eluted in

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[3] The abbreviations used are: MSRF, methylation-sensitive restriction fingerprinting; HBC, hypermethylation in breast cancer; RLGS, restriction landmark genomic scanning.

water and reamplified by 30 cycles of PCR with appropriate primers. Unmodified PCR products were ligated directly into the TA cloning vector, pCR II, and transformed into *Escherichia coli* competent cells following the supplier's protocol (Invitrogen). Six to eight white colonies were selected, and plasmid DNA was isolated using minipreparation protocols (Qiagen). Double-stranded plasmid DNA was prepared and sequenced with an automated ABI model 373 sequencer (Applied Biosystems, Inc.). The resulting nucleotide sequence was compared to GenBank using the BLAST program (14).

Southern Hybridization Analysis. Five μg of genomic DNA were digested with *Mse*I alone or digested with *Mse*I and *Bst*U I at high concentration (15 units/μg) to ensure complete digestion of both tumor and normal DNAs. The digestion products were separated on a 1.5% agarose gel and transferred to nylon membrane (Schleicher & Schuell). Filters were hybridized with a ^{32}P-labeled HBC-1 probe (230 bp) or a ^{32}P-labeled fragment, HBC-2.1 (119 bp; positions 21–139; see Fig. 2*B*). Hybridization and washing conditions were carried out essentially as described (15). Filters were exposed to Kodak BioMax film in the presence of an intensifying screen for 7 days at −70°C.

Results and Discussion

Several fingerprinting techniques based on arbitrarily primed PCR (16, 17) have previously been applied to identify genomic alterations (18) and differentially expressed transcripts (19) in several types of tumor cells. In the present report, we describe an improved fingerprinting technique, MSRF, to identify abnormally methylated CpG sites in the breast tumor genome. Three unique features have been implemented to preferentially analyze methylation changes within CpG islands: (*a*) the system used a 4-base cutter, *Mse*I, which has previously been shown to restrict genomic DNA into small fragments (once per 140 bp) but preserve the integrity of the CpG islands (once per 1,000 bp; Ref. 20); (*b*) the methylation-sensitive endonuclease *Bst*U I was used for differentiating CpG methylation between tumor and normal genomes. This endonuclease was chosen because its recognition sequence (CGCG) occurs frequently within CpG islands (once per 90 bp) but is rare in bulk DNA (one site per 5,000–10,000 bp) (20); and (*c*) methylated CpG islands protected from the *Bst*U I digestion were PCR-amplified with short arbitrary primers (10-mers) attaching to the flanking homologous sequences in the genome in opposite directions. Except for primer Bs18 (see "Materials and Methods"), these primers were designed further to contain CGCG at their 3′ ends, which would enhance the chance of amplifying regions harboring the *Bst*U I sites.

At least four conditions related to DNA methylation are distinguished by MSRF (Fig. 1*A*). In a condition showing no methylation (condition 1), PCR products are identified only in the *Mse*I-digested tumor and normal DNAs (Fig. 1 *A*, *Lanes 2* and *4*) but not in the *Mse*I/*Bst*U I-digested tumor and normal DNAs (Fig. 1 *A*, *Lanes 1* and *3*). The absence of products in Lanes 1 and 3 (Fig. 1*A*) occurs because the given genomic sequence, which contains an internal CGCG site(s), is restricted with *Bst*U I and cannot be amplified by PCR using appropriate primers. In condition 2, an internal CGCG site(s) for a given genomic sequence is methylated in both tumor and normal genomes and thus is protected from the *Bst*U I digestion. Alternatively, the given genomic sequence does not contain an internal *Bst*U I recognition sequence(s). In either situation, amplified products are present in all four lanes (Fig. 1*A*). An aberrant hypermethylation event (condition 3) occurs in tumor when an amplified product is observed (or shows a relative increase in band intensity) in the *Mse*I/*Bst*U I-digested tumor DNA (Fig. 1*A*, *Lane 1*) but not in the double-digested normal DNA (Fig. 1*A*, *Lane 3*). Amplified products are seen in the *Mse*I-digested tumor and normal DNAs (Fig. 1*A*, *Lanes 2* and *4*). This indicates that a *Bst*U I site(s) is specifically methylated in tumor DNA, but not in normal DNA. Hypomethylation (condition 4) is detected in tumor when a PCR product is observed in the double-

A

Methylation conditions in tumor DNA relative to normal DNA	Gel lanes: Tumor DNA *Mse* I/*Bst*U I (1)	Tumor DNA *Mse* I (2)	Normal DNA *Mse* I/*Bst*U I (3)	Normal DNA *Mse* I (4)
1. No methylation		—		—
2. Normal methylation (or no *Bst*U I sites)	—	—	—	—
3. Hypermethylation				
a. Complete	—	—		—
b. Enhanced	—	—	—	—
4. Hypomethylation				
a. Complete		—	—	—
b. Incomplete	—	—	—	—

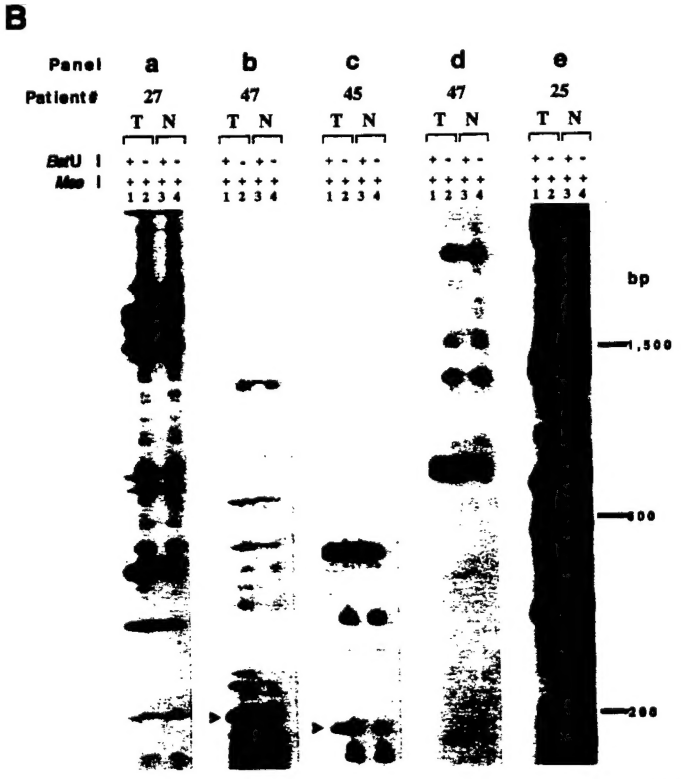

Fig. 1. MSRF. *A*, possible outcomes of DNA methylation changes in tumor detected by MSRF. *B*, genomic DNAs from breast tumor (*T*)/normal (*N*) tissue pairs were treated (+) with *Mse*I. The digests were further treated (+) or untreated (−) with *Bst*U I. Patient numbers are shown above *lanes*. The digests were separately amplified with primer pairs Bs7/Bs10 (*a*), Bs1/Bs18 (*b*), Bs12/Bs13 (*c*), Bs13/Bs17 (*d*), and Bs11/Bs5 (*e*). The amplified products were size fractionated on 4.5% polyacrylamide gels. Patient numbers are shown above *lanes*. Molecular weight markers (100-bp ladder; Life Technologies, Inc.) are shown to the right. *Arrowheads*, hypermethylation-containing DNA fragments in tumor DNA.

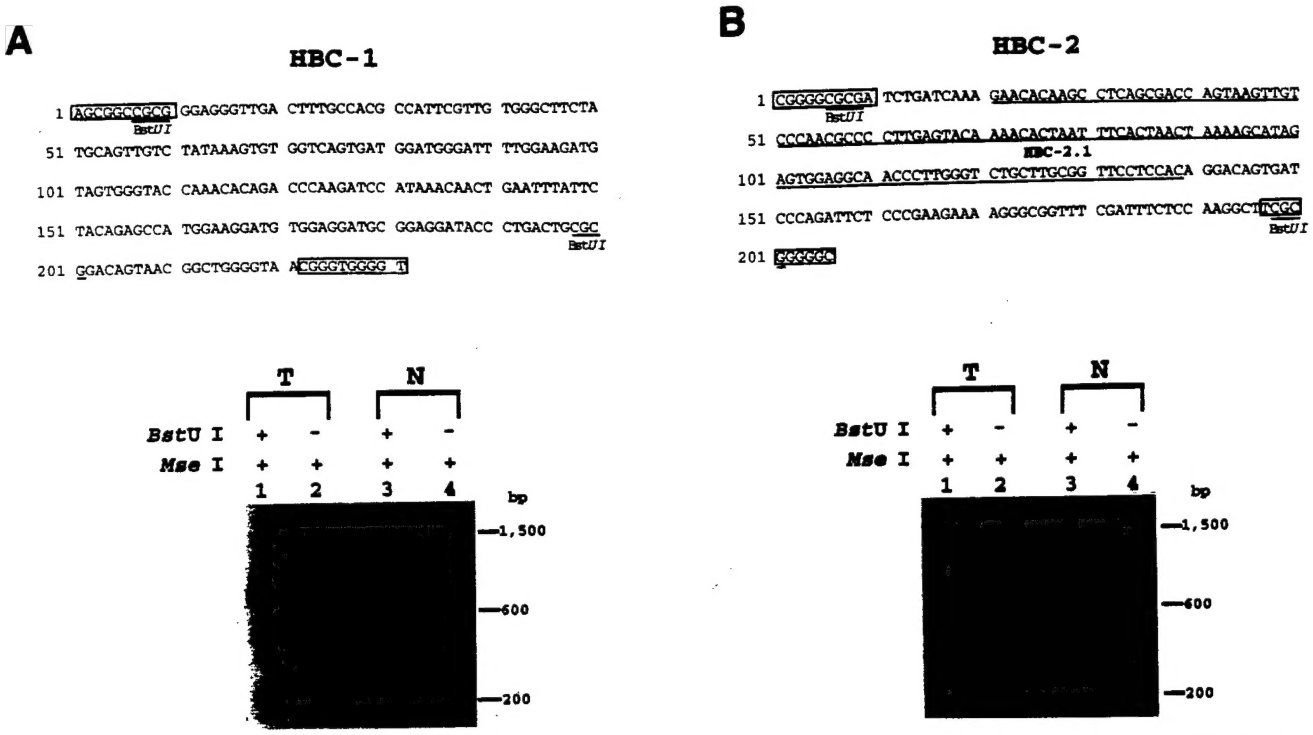

Fig. 2. *A* and *B*, *upper panels*, nucleotide sequences of HBC-1 and HBC-2. Both strands of the inserts were sequenced with T7 and SP6 promoter primers. Primers used in MSRF are shown in the boxed regions. The restriction endonuclease sites for *Bst*U I are underlined. An internal fragment, HBC-2.1(underlined), was used as a probe in Southern hybridization; *lower panels*, methylation analysis of breast tumor-normal DNA pairs by Southern hybridization. Genomic DNA (5 μg) was treated (+) or untreated (−) with restriction endonucleases as indicated and subjected to Southern hybridization analysis using HBC-1 and HBC-2.1, respectively, as probes. *T*, breast tumor, *N*, normal breast tissue. Molecular weight markers (100 bp ladder; Life Technologies, Inc.) are shown to the right.

digested normal DNA (Fig. 1*A*, *Lane 3*), but not (or in a reduced quantity) in the double-digested tumor DNA (Fig. 1*A*, *Lane 1*).

Representative results of MSRF screening using a panel of primer pairs are shown in Fig. 1*B*. The majority of amplified products showed either condition 1 (no methylation) or condition 2 (normal methylation or no internal *Bst*U I sites). Two candidate fragments containing abnormally hypermethylated *Bst*U I sites (condition 3) in the tumor DNA were observed in Fig. 1*B*, *b* and *c* at ~230 and ~200 bp, respectively (Fig. 1*B*, *arrowheads*); these DNA bands were detected in the *MseI/Bst*U I-digested tumor DNA, but not in the double-digested normal DNA. Methylation differences between amplified tumor and normal DNAs were not noted in Fig. 1*B*, *a*, *d*, or *e*. PCR products were not observed in the control reactions (no DNA template) amplified with these primer pairs at the concentration specified in the protocol.

The two hypermethylation-containing fragments (~230 and ~200 bp), now designated as HBC-1 and HBC-2, respectively, were cloned for sequencing. The fragments were found to have the expected primers at the 5′ and 3′ ends (Fig. 2, *A* and *B*, *top*). A BLAST search revealed no significant matches between the known sequences in the database and the sequence of HBC-1. Subsequent observation has shown that HBC-1 contains a novel expressed sequence based on a Northern hybridization analysis.[4]

Studies are currently underway to clone the full-length cDNA related to HBC-1. HBC-2 matched the upstream region of the antisense WT1 (Wilms' tumor suppressor gene) promoter located in the first intron on the chromosome 11p13 region; the 3′ end of HBC-2 at position 157–206 matched the published sequence of the antisense WT1 promoter at position 1–50 (GenBank accession no. S79781; Ref. 21), while the rest matched the unpublished sequence.[5]

We further confirmed the findings of aberrant hypermethylation in breast tumors by Southern hybridization analysis. The HBC-1 probe detected an ~800-bp band (Fig. 2*A*, *Lanes 2* and *4*) in the *Mse*I-digested tumor and normal DNAs from patient 47. HBC-1 also detected an ~800-bp fragment in the *MseI/Bst*U I-digested breast tumor DNA from patient 47 (Fig. 2*A*, *Lane 1*) but hybridized to a band of a smaller size (~190 bp) in the digested normal DNA of the same patient (Fig. 2*A*, *Lane 3*). This result indicates that the *Bst*U I sites located within the ~800 bp *Mse*I-digested fragment were protected (*i.e.*, methylated) from restriction in the tumor DNA. The ~190-bp band detected corresponds to the *Bst*U I-restricted fragment within the HBC-1 sequence, indicating that the two *Bst*U I sites were unmethylated in the normal DNA. (A minor band at ~190 bp was observed at the *MseI/Bst*U I-digested tumor DNA, which can be attributed to the presence of contaminating normal tissue in the tumor sample.) A similar Southern hybridization result was obtained when HBC-2.1 (an internal fragment of HBC-2 from the antisense WT1 promoter) was used as a probe (Fig. 2*B*, *bottom*). The *Bst*U I sites located within the ~1000 bp *Mse*I-digested fragment were found to be methylated in the digested tumor DNA (Fig. 2*B*, *Lane 1*). HBC-2 detected two fragments (~200 and ~500 bp) in the digested normal DNA (*Lane 3*); the ~200-bp fragment corresponds to the *Bst*U I-digested fragment within HBC-2, indicating that the two *Bst*U I sites were unmethylated in the normal DNA, whereas the ~500-bp fragment can be attributed to the presence of partially methylated *Bst*U I sites in some normal cells.

We extended the methylation analysis of HBC-1 to an additional 18 breast tumor-normal DNA pairs. Fig. 3*A* shows representative results of four patients using MSRF. The HBC-1 fragment was detected in the amplified double-digested tumor DNA of patients 19, 25, and 65, but not in that of patient 31. HBC-1 was not noted in the double-digested normal

[4] T. H-M. Huang, unpublished data.
[5] K. T. A. Malik, personal communication.

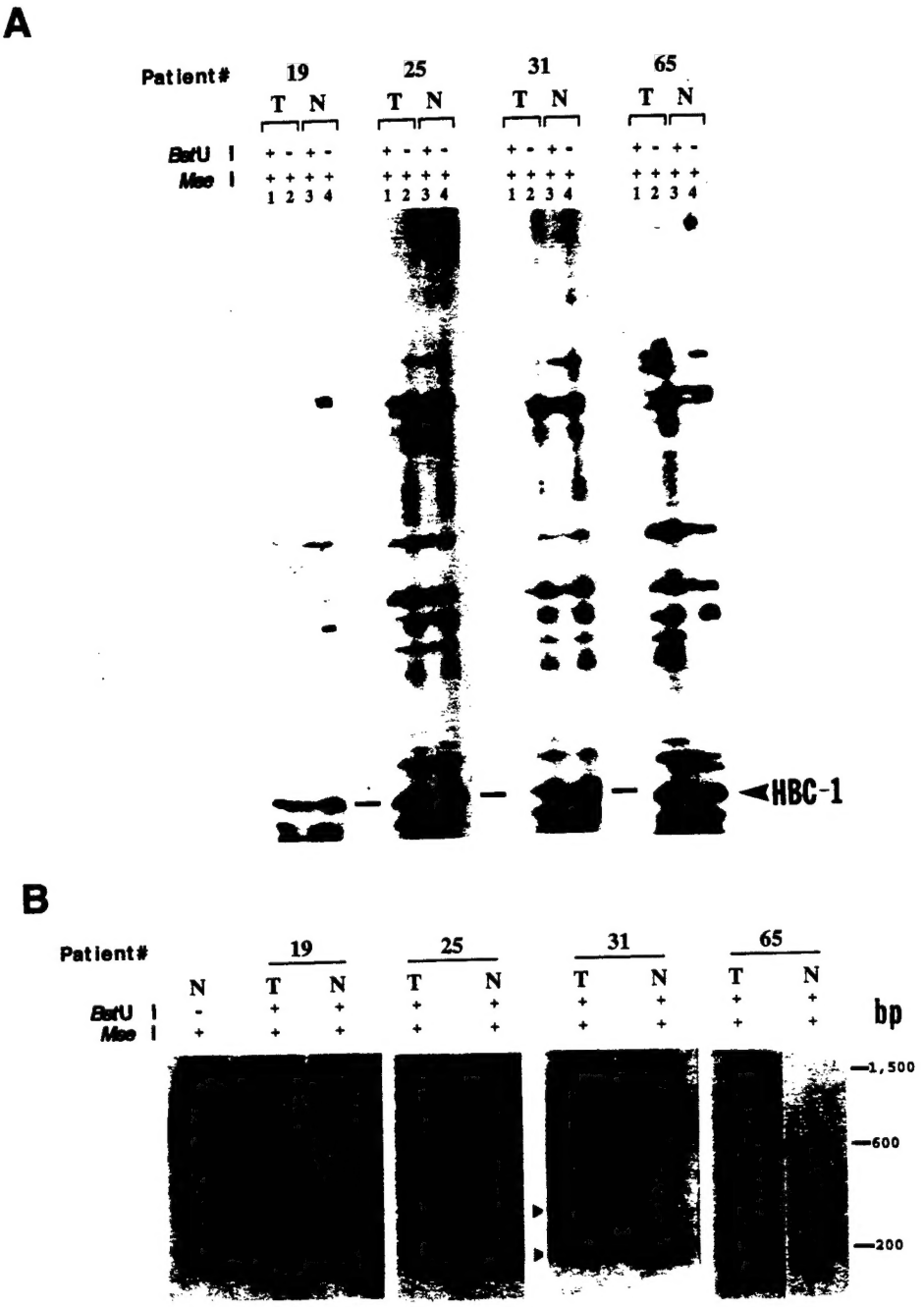

Fig. 3. Representative results of methylation-sensitive restriction fingerprinting (MSRF) and Southern hybridization. *A*, Genomic DNA from breast tumor (*T*)/normal (*N*) tissue pairs of patients was treated (−) or untreated (+) with restriction endonucleases as indicated. The digests were amplified with a primer pair (Bs1 and Bs18). The amplified products were size-fractionated on 4.5% polyacrylamide gels. Patient numbers are shown at the top of lanes. Molecular weight markers (100-bp ladder; Life Technologies, Inc.) are shown to the right. *Arrowheads*, the location of a hypermethylation-containing fragment HBC-1 (see explanation in Fig. 1A). *B*, Genomic DNA (5 μg) was treated (+) or untreated (−) with restriction endonucleases as indicated and subjected to Southern hybridization analysis using HBC-1 as a probe. *T*, breast tumor, *N*, normal breast tissue. Patient numbers are shown at the top of lanes. Molecular weight markers (100 bp ladder; Life Technologies, Inc.) are shown to the right.

DNA of these patients (Residual intensities of bands seen in the double-digested normal DNA could be due to incomplete restriction of *Bst*U I or low-level methylation). A parallel Southern hybridization analysis (Fig. 3*B*) was also conducted, showing a consistent finding of hypermethylation detected in the tumor DNA of patients 19, 25, and 65. The digested tumor DNA of patient 31 showed at least two smaller fragments (Fig. 3 *B, arrowheads*), ~200 and ~400 bp, that can be attributed to partially methylated or unmethylated *Bst*U I sites within the *Mse*I-restricted fragment. This condition may reflect the heterogeneous nature of this primary tumor or contamination of normal cells in the tumor specimen analyzed. The methylation data based on Southern hybridization as well as patients' clinical information are summarized in Table 1. Taken together, HBC-1 was methylated aberrantly in 90% (17 of 19 patients) of the primary breast tumors examined. Currently, we are studying more tumor samples to determine whether hypermethylation of HBC-1 is correlated with specific clinicopathological parameters of the patients with breast cancer.

Hypermethylation of HBC-2 within the antisense promoter in the intron 1 of WT1 also appeared to be common in primary breast carcinomas.[4] We are currently extending the methylation study to the promoter and first exon regions of WT1. The results will be reported in a separate paper.

Several conditions may interfere with identification of true abnormal methylation sites from breast tumors. As discussed by others, artifacts occur in the PCR-based methodologies such as representational difference analysis (22) or differential display (23). As shown in Fig. 3*A*, this potential problem can be minimized by studying independent tumors and by scoring the same aberrant PCR products only consistently revealed in a group of patients. Secondly, because

Table 1 *Clinical information and methylation studies of female patients with breast tumors*

Patient no.	Age at diagnosis (yr)	Histological type[a]	Clinical staging[b]	Methylation status of HBC-1 in tumor[c]
3	64	MD, IDC	IIIb	+
9	64	MD, IDC	IIb	+
19	64	MD, IDC	IV	+
25	42	MD, IDC	IIIa	+
27	50	MD, IDC	IIIb	+
29	77	WD, IDC	ND	+
31	60	PD, IDC	IIb	−
33	40	Lobular	IIIa	+
43	35	PD, IDC	IIIb	−
45	44	MD, IDC	IIIa	+
47	38	PD, IDC	IIIa	+
65	42	PD, IDC	IIIa	+
67	40	MD, IDC	IIIb	+
71	33	MD, IDC	IIa	+
73	56	MD, IDC	IIa	+
79	48	MD, IDC	IIa	+
83	45	MD, IDC	IIIa	+
87	76	MD, IDC	IIa	+
89	83	PD, IDC	IV	+

[a] WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; IDC, infiltrating ductal carcinoma.

[b] Clinical staging was according to the criteria of the American Joint Committee on Cancer (27). ND, not determined.

[c] +, hypermethylation in tumor; −, lack of methylation in tumor (see additional description in the text).

primary tumor specimens rather than homogeneous cell lines were used in our MSRF screening, residual normal tissue is likely to be present in the cancer biopsies, or *vice versa*. Nevertheless, the residual tissue contamination problem does not apply to detecting the gain of PCR fragments containing hypermethylated CpG sequences in tumor specimens that have some normal cells present.

MSRF also can be applied to detect hypermethylated DNA in specimens with multicenter or multifocal lesions, in which it is not possible to obtain pure tumor cells. However, the detection of hypomethylation-containing fragments by MSRF can be impaired if contaminating normal cells are present in the tumor tissue to be tested. Because a small amount of genomic DNA (20–100 ng) is applied in MSRF, one future improvement would be the use of microdissection techniques (24), which allows the isolation of pure cell populations.

To our knowledge, only one other technique, RLGS, has previously been developed for screening changes of CpG methylation between different cell types (25, 26). RLGS employed digestion of genomic DNA with a series of restriction endonucleases, one being methylation sensitive *Not*I. The digests are then subjected to two rounds of gel electrophoresis, generating complicated two-dimensional DNA patterns. Identification of methylation differences is based on comparison of more than 2500 *Not*I spots between two autoradiographs representing two different cell types. The procedure had the advantage of surveying thousands of CpG islands in a single experiment. The limitation of RLGS is that a substantial amount of genomic DNA is required to start with, to enable the subsequent cloning of differential spots. For example, 500 μg of genomic DNA was used by Kawai *et al.* (25) in RLGS to clone methylation-specific DNA spots for different stages of brain development in the mouse.

MSRF, described here, provides an additional approach to study CpG methylation throughout the genome. The procedure is PCR based, only needs a small amount of genomic DNA, and, thus, is suitable for analyzing clinical specimens. DNA of interest is isolated directly from gels and reamplified by PCR for cloning. Additionally, it should be possible to speed up methylation screening with more short primers by a high-throughput protocol using a 96-well plate format thermocycler. Therefore, it is expected that MSRF should have a wide-ranging application in the study of changes in DNA methylation in cancer as well as in aging, cell differentiation, and development.

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References

1. Baylin, S. B., Makos, M., Wu, J. J., Yen, R. W. C., de Bustros, A., Vertino, P., and Nelkin, B. D. Abnormal patterns of DNA methylation in human neoplasia: potential consequences for tumor progression. Cancer Cells (Cold Spring Harbor), *3:* 383–390, 1991.
2. Jones, P. A. DNA methylation errors and cancer. Cancer Res., *56:* 2463–2467, 1996.
3. Adam, R. L. P., and Burdon, R. H. Molecular biology of DNA methylation. New York: Springer-Verlag New York, Inc., 1985.
4. Ottaviano, Y. L., Issa, J-P., Parl, F. F., Smith, H. S., and Baylin, S. B. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res., *54:* 2552–2555, 1994.
5. Huynh, L., Alpert, L., and Pollak, M. Silencing of the mammary-derived growth inhibitor (MDGI) gene in breast neoplasms is associated with epigenetic changes. Cancer Res., *56:* 4865–4870, 1996.
6. Lee, W-H., Morton, R. A., Epstein, J. I., Brook, J. D., Campbell, P. A., Bova, G. S., Hsieh, W. S., Isaacs, W. B., and Nelson, W. G. Cytidine methylation of regulatory sequences near the π-class *glutathione S-transferase* gene accompanies human prostatic carcinogenesis. Proc. Natl. Acad. Sci. USA, *91:* 11733–11737, 1994.
7. Herman, J. G., Latif, F., Weng, Y., Lerman, M. I., Zbar, B., Liu, S., Samid, D., Duan, D-S. R., Gnarra, R. J., Linehan, W. M., and Baylin, S. B. Silencing of the *VHL* tumor-suppressor gene by DNA methylation in renal carcinoma. Proc. Natl. Acad. Sci. USA, *91:* 9700–9704, 1994.
8. Sakai, T., Toguchida, J., Ohtani, N., Yandell, D. W., Rapaport, J. M., and Dryja, T. P. Allele-specific hypermethylation of the *retinoblastoma* tumor-suppressor gene. Am. J. Hum. Genet., *48:* 880–888, 1991.
9. Wales, M. M., Biel, M. A., El Deiry, W., Nelkin, B. D., Issa, J-P., Cavenee W. K., Kuerbitz, S. J., and Baylin, S. B. p53 activates expression of HIC-1, a new candidate tumor suppressor gene on 17p13.3. Nat. Med., *1:* 570–577, 1995.
10. Herman, J. G., Merlo, A., Mao, L., Lapidus, R. G., Issa, J-P. J., Davidson, N. E., Sidransky, D., and Baylin, S. B. Inactivation of the *CDKN2/p16/MTS1* gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res., *55:* 4525–4530, 1995.
11. Gonzalez-Zulueta, M., Bender, C. M., Yang, A. S., Nguyen, T., Beart, R. W., Van Tornout, J. M., and Jones P. A. Methylation of the 5′ CpG island of the *p16/CDKN2* tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res., *55:* 4531–4535, 1995.
12. Yoshiura, K., Kanai, Y., Ochiai, A., Shimoyama, Y., Sugimura, T., and Hirohashi, S. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc. Natl. Acad. Sci. USA, *92:* 7416–7419, 1995.
13. Ponce, M. R., and Micol, J. L. PCR amplification of long DNA fragments. Nucleic Acids Res., *20:* 623, 1992.
14. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. Basic local alignment search tool. J. Mol. Biol., *215:* 403–410, 1990.
15. Huang, T. H-M., Hejtmancik, J. F., Edwards, A., Pettigrew, A. L., Herrera, C. A., Hammand, H. A., Caskey, C. T., Zoghbi, H. Y., and Ledbetter, D. H. Linkage of the gene for an X-linked mental retardation disorder to a hypervariable (AGAT)n repeat motif within the human HPRT locus (Xq26). Am. J. Hum. Genet., *49:* 1312–1319, 1991.
16. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., *18:* 6531–6535, 1990.
17. Welsh, J., and McClelland, M. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res., *18:* 7213–7218, 1990.
18. Peinado, M. A., Malkhosyan, S., Velazquez, A., and Perucho, M. Isolation and characterization of allelic losses and gains in colorectal tumors by arbitrarily primed polymerase chain reaction. Proc. Natl. Acad. Sci. USA, *89:* 10065–10069, 1992.
19. Liang, P., and Pardee, A. B. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science (Washington DC), *257:* 967–971, 1992.
20. Cross, S. H., Charlton, J. A., Nan, X., and Bird, A. P. Purification of CpG islands using a methylated DNA binding column. Nat. Genet., *6:* 236–244, 1994.
21. Malik, K. T. A., Wallace, J. I., Ivins, S. M., and Brown, K. W. Identification of an antisense WT1 promoter in intron 1: implication for *WT1* gene regulation. Oncogene, *11:* 1589–1595, 1995.
22. Lisitsyn, N., Lisitsyn, N., and Wigler, M. Cloning the differences between two complex genomes. Science (Washington DC), *259:* 946–951, 1993.
23. Watson, M. A., and Fleming, T. P. Isolation of differentially expressed sequence tags from human breast cancer. Cancer Res., *54:* 4598–4602, 1994.
24. Zheng, Z., Merino, M. J., Chuaqui, R., Liotta, L. A., and Emmert-Buck, M. R. Identical allelic loss on chromosome 11q13 in microdissected *in situ* and invasive human breast cancer. Cancer Res., *55:* 467–471, 1995.
25. Kawai, J., Hirotsune, S., Hirose, K., Fushiki, S., Watanabe, S., and Hayashizaki, Y. Methylation profiles of genomic DNA of mouse developmental brain detected by restriction landmark genomic scanning (RLGS) method. Nucleic Acids Res., *21:* 5604–5608, 1993.
26. Hatada, I., Sugama, S., and Mukai, T. A new imprinted gene cloned by a methylation-sensitive genome scanning method. Nucleic Acids Res., *21:* 5577–5582, 1993.
27. American Joint Committee on Cancer. Manual for Staging of Cancer, pp. 93–99. Philadelphia: J. B. Lippincott Co., 1989.